

***Hyalella azteca* 10-Day Water Toxicity Test**

1.0 OBJECTIVE

In laboratory tests designed to determine the toxicity of low-salinity water samples, *Hyalella azteca* are exposed to test solutions for 10 days, after which the percentage mortality is determined. Observed effects may be related to the presence of contaminants or to naturally occurring factors. In order to correctly interpret toxicity results, concentrations of chemical contaminants should be analyzed, as well as other water quality parameters, such as dissolved oxygen, pH, conductivity, ammonia, hardness, alkalinity, and temperature.

In this procedure, water samples collected from field stations are divided into randomly numbered replicate test containers in the laboratory. Ten *H. azteca* are placed into each replicate container. Each beaker is monitored daily for mortality, and is renewed at 5 days. After a 10-day exposure, survival is counted and recorded to give an estimate of sample toxicity. Because the test measures effects on an early life-stage of an ecologically important species possessing relatively stringent water quality requirements, the results constitute a good basis for decisions concerning either hazard evaluation or the suitability of estuarine waters for aquatic life (U.S. EPA 2002).

2.0 EQUIPMENT

The following equipment is necessary to conduct the toxicity test at the Marine Pollution Studies Laboratory at Granite Canyon (MPSL). The word "clean" here and throughout this procedure means that the item has been cleaned according to the MPSL glassware cleaning procedures outlined in a separate standard operating procedure (MPSL SOP 1.3).

2.1 Culture

- Pipettes, tubing, and clean air system
- Short buckets or trays
- Granite Canyon well water ($23 \pm 1^{\circ}\text{C}$)
- YCT for feeding, purchased from Aquatic Biosystems (Fort Collins, CO)
- *Hyalella azteca* (7-14 days old, supplied by Chesapeake Cultures (Gloucester, VA))

2.2 Test Initiation/Termination

- Environmental chamber ($23 \pm 1^{\circ}\text{C}$, ambient laboratory illumination for 16 hours/day)
- 300-mL clean glass beakers (5 per sample)
- 250-mL clean plastic tri-pour beakers (3 per reference toxicant concentration)
- Clear plastic sheets to cover beakers
- 1000-mL volumetric flask for reference toxicant concentrations
- 10-mL and micro-pipettors and pipettes for reference toxicant concentrations
- Cadmium chloride stock solution (100,000 $\mu\text{g/L}$ Cd)
- Randomization sheet to arrange and identify test containers

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- Data sheets
- Gloves and appropriate safety gear (see MPSL lab safety manual)
- Sample vials for reference toxicant analysis (new polyethylene 30 mL, acid washed)
- Dissecting microscope for counting neonates
- Disposable plastic pipettes with cut-off tips (for handling animals)

2.3 Water Quality

- Meters, probes, spectrophotometer, digital titrator and standards for measuring pH, dissolved oxygen, hardness, alkalinity, ammonia, and conductivity
- Thermometers (glass spirit thermometer and continuously recording thermometer)
- Graduated pipettes (10 mL) and hand pipette pump for water quality sampling
- Water quality vials (30 mL glass)
- Gloves and appropriate safety gear (see MPSL lab safety manual)

2.4 Dilution Water

In every step of this procedure, use Granite Canyon well water.

3.0 EXPERIMENTAL DESIGN

Aquatic toxicity tests can be used as screening tools or as part of more comprehensive studies to assess water quality. Careful consideration must be given to site characteristics, reference site selection, field replication, choice of synoptic measures, seasonal factors, and comprehensive planning and peer review to determine that study designs are adequate to meet program objectives.

This laboratory toxicity test consists of five replicate test beakers for each sample concentration. Beakers are arranged randomly, and each receives ten *H. azteca*. The quality of test animals and testing conditions is determined through concurrent testing of reference toxicants (positive controls) and control water (negative controls). Testing of reference sites or receiving water is recommended to demonstrate the suitability of test sites in the absence of toxic contaminant concentrations. Test conditions of temperature and photoperiod are controlled as indicated below, and dissolved oxygen, pH, conductivity, and ammonia are measured at the beginning and end of the exposure. Temperature is measured continuously, and hardness and alkalinity are measured at the beginning of the test.

4.0 PREPARATION OF SAMPLES FOR TESTING

One day before test initiation, the volume necessary for test initiation should be placed in the constant temperature room (23°C) to allow oxygen concentrations to equilibrate below super-saturated levels; the minimum time should

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be 12 hours. Prepare five replicate 300-mL beakers for each sample to be tested. Consult the random number sheet to ensure proper randomization. Each container receives 100 mL of test solution.

5.0 CONTROLS

5.1 Dilution Control

The dilution control should consist of Granite Canyon well water.

5.2 Reference Toxicant Tests

For cultured organisms, conduct a concurrent reference toxicant at least monthly. The reference toxicant test indicates the sensitivity of the organisms and the suitability of the test methodology.

Reagent grade cadmium chloride (CdCl_2) should be used as the reference toxicant for *H. azteca* tests, unless another toxicant is specified. Prepare a 100,000 $\mu\text{g/L}$ Cd stock solution by adding 0.1630 g of reagent grade CdCl_2 to a final volume of one liter of distilled water in a plastic volumetric flask. Cap tightly and mix thoroughly. Sample and log the reference toxicant stock solution at the beginning of the test for chemical verification of the copper concentration. Acidify samples for analysis in clean sample vials with 1% by volume 14N-reagent grade nitric acid..

Reference toxicant solutions should be three to five replicates of 0 (control), 5.6, 10, 18, 32, and 56 $\mu\text{g Cd/L}$. Other concentrations may be added between these if greater precision is desired for quality control chart purposes. Prepare 1000 mL of each concentration by adding stock solution (see dilution schedule) to a 1000-ml plastic volumetric flask and fill with culture water. Aliquot each concentration to randomly numbered test containers as indicated on the random number sheet, and into water quality vials. Start with the control solutions and progress to the highest concentration to minimize contamination. Place the reference toxicant test containers in the constant temperature room, cover, and equilibrate.

All tests (sample and reference toxicant) must use *H. azteca* from the same culture. They must be handled in the same way and delivered to the test containers at the same time.

6.0 TEST ORGANISMS

6.1 Laboratory Acclimation

Order amphipods to arrive between two and seven days before test initiation. Amphipods must be between 7 and 14 days old at test initiation. Place the amphipods in a culture tray containing well water at a temperature that varies by no more than 3°C from transport conditions. Acclimate the amphipods to test temperature and conductivity. Hold amphipods at test temperature for 48 hours prior to initiating sediment testing. Remove any dead or moribund animals. Make sure water in the tray is constantly aerated. Check the amphipods daily, and monitor the health of

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amphipods by observing appearance. If more than 5% of the amphipods appear unhealthy during the 48 hours prior to the test, reschedule the test and immediately arrange for another amphipod shipment. Renew the culture daily before the test with dilution water, and feed 10 mL YCT daily.

6.2 Amphipod Loading

Using a clean transfer pipette with the tip removed. Transfer the amphipods from the culture tray into the test containers. Only transfer animals that are healthy and moving. Replace injured or stressed amphipods. Continue until each container has 10 animals. Maintain water temperature ($23 \pm 1^\circ\text{C}$) by sorting animals in the constant temperature room where the test is being held.

7.0 MONITORING THE TOXICITY TEST

7.1 Counting *Hyalella* Mortality

Test duration is 10 days. Check all test containers daily, and record the number of live animals. Also attempt to count the number dead to ensure that the total number of animals in the container at the start of the test was 10; if not, record this on the data sheet. Immobile *H. azteca* that do not respond to a stimulus are considered dead. The stimulus should be a gentle stream of water from a disposable pipette. *H. azteca* that exhibits a visible response is considered living. Remove dead animals.

7.2 Measuring Water Quality in Test Containers

Temperature, dissolved oxygen, pH, ammonia, and conductivity are measured at test initiation and termination. Hardness and alkalinity should be measured at the beginning of the test. Sample the initial test solutions at the time of dilution preparation. Water quality should also be measured on old and new dilutions at the time of renewal. Renewal water quality parameters include dissolved oxygen, pH, conductivity, and temperature. Water quality should be measured only initially on reference toxicant tests.

7.3 Feeding

Test containers are fed 0.5 mL YCT every other day starting at Day 0. Reference toxicant containers are fed 0.5 mL YCT on Day 0 and Day 2.

8.0 TEST SOLUTION RENEWAL

The test duration is 10 days. Because toxicity may change over short periods in test containers, the test solutions must be renewed after 5 days. Prepare new test solutions as in section 4. One day before solution renewal, the volume necessary for renewal should be placed in the constant temperature room (23°C) to allow oxygen concentrations to equilibrate below super-saturated levels. These samples must be aerated if dissolved oxygen concentrations exceed maximum values allowed.

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At renewal time make survival observations then remove ~80% of old test solutions reserving enough for water quality measurements. Old test solutions can be removed by siphon, taking care not to remove any organisms. Introduce new test solutions by gently pouring down the side of the beaker or down a glass rod to minimize stress to animals. Measure dissolved oxygen on the new sample, and dissolved oxygen, pH, and ammonia on the old sample.

9.0 TERMINATING THE TOXICITY TEST

After 10 days of exposure, final mortality counts are made.

Final water quality must be sampled at the termination of the test. Deliver a sample from each site into pre-labeled water quality containers. Measure and record temperature, dissolved oxygen, pH and conductivity of each sample. Only temperature is measured for the reference toxicant test.

Take the completed data sheet to the office for data entry and analysis. Notify the data analyst that the data has arrived. Make sure the data sheets are placed in the proper location and that the person keeping track of the data knows where it is.

10.0 DATA HANDLING AND TEST ACCEPTABILITY

Immediately after test termination, determine whether dilution water and conductivity controls have acceptable survival ($\geq 90\%$). If not, notify the project officer without delay. Tests with temperature, salinity, or dissolved oxygen measurements outside the specified ranges, may be considered conditionally acceptable based on the project officer's best professional judgment. Acceptable temperatures are $23 \pm 1^\circ\text{C}$; acceptable dissolved oxygen concentration is 60-100% saturation.

11.0 REFERENCES

US EPA. 2002. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. U.S. Environmental Protection Agency, Office of Research and Development. EPA-821-R-02-012.

12.0 TEST SUMMARY

Species:	<i>Hyalella azteca</i>
Test Duration:	10 Days
Endpoint:	Survival
Renewals:	80% on Day 5
Organism Source	Chesapeake Cultures (Gloucester, VA)
Age of Test Organisms:	7-14 Days
Salinity Range:	0-15 ‰ recommended

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Dissolved Oxygen	>4 mg/L recommended
Temperature:	23 ± 1°C recommended
Dilution Water:	Granite Canyon well water
Light intensity:	Ambient laboratory illumination (10-20 $\mu\text{E}/\text{m}^2/\text{s}$)
Photoperiod:	16 hour light: 8 hour dark
Replication:	5 (samples), 3 (reference toxicant)
Test Containers:	300-mL glass beakers
Test Solution Volume:	100 mL
Loading:	10 animals per beaker
Feeding:	In culture prior to test initiation and 0.5 mL YCT every other day
Water Quality:	pH, dissolved oxygen, temperature, conductivity, hardness, alkalinity, ammonia
Reference Toxicant:	cadmium chloride (CdCl_2)
Daily Monitoring:	count alive and remove dead
Acceptability Criteria:	mean survival in dilution water controls $\geq 90\%$